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EFFECT OF SULFATION ON THE STRUCTURE AND ANTICOAGULANT ACTIVITY OF ULVAN EXTRACTED FROM GREEN SEAWEED *ULVA RETICULATA*

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ABSTRACT

Ulvans are sulfated polysaccharides derived from the cell wall of green seaweeds. The chemical structure of ulvan extracted from *Ulva reticulata* is reported, focusing on investigation of the sulfated modification of the ulvan and the changes in structure and anticoagulant activity. The results showed that sulfated modification was able to change the ulvan conformational structure and markedly enhance its anticoagulant activity.

Keywords: ulvan, green seaweed, *Ulva reticulata,* sulfation, structure, anticoagulant activity.

1. INTRODUCTION

Ulvans are water-soluble sulfated polysaccharides derived from marine green seaweeds. They were reported to exhibit a wide range of physiological and biological activities such as anticancer, anticoagulant, antioxidant, antifungal and antitumor activities. Ulvan essentially contains rhamnose, xylose, glucuronic acid, iduronic acid and sulfate groups [1, 2, 3]. The chemical composition and structure of ulvan may vary depending on the algae species, place of cultivation and method of extraction [4, 5].

Green seaweed family has many species including *Ulva lactuca, Ulva pertusa*, *Ulva rigida*, *U. rotundata, U. reticulata, Enteromorpha intestinalis, E. Compressa...*Among them, *Ulva lactuca* and *Ulva reticulata* are more popular [6]. In previous paper [7], we reported the results on extraction and structural determination of ulvan from green seaweed *Ulva reticulata*. Many previous researches reported that biological activity of sulfated polysaccharides depends on the content of sulfate groups and sulfation modification may lead to the change on structure and bioactivity of the polysaccharides [8, 9]. Therefore, in this paper, sulfated derivative of ulvan extracted from green seaweed *Ulva reticulata* was prepared and anticoagulant activity *in vitro*

was estimated in order to look at the effect of sulfation on structure and anticoagulant activity of the ulvan.

2. EXPERIMENT

2.1. Material

Chemicals: Sodium bisulfite, sodium nitrite and other reagent used were of analytical grade from Shantou Xilong Chamical Factory Guang Dong China without further purification.

Ulvan extraction: The extraction was followed the method of Wenjun Mao et al. [10]. 20 g of dry seaweed in ethanol 80 % was kept over night to remove colored and low molecular weight compounds and then dried. The obtained alga per 400 mL of water was heated for of 2 h at 80–90 ºC on a boiling-water bath under continuous stirring. The aqueous extract was centrifuged, and the liquid supernatant was filtered. The water extract was concentrated to reduce initial volume in a rotary evaporator and precipitated with 4 vol. of absolute ethanol. The alcohol precipitate was separated by centrifugation to obtain ulvan. Obtained ulvan was dissolved in water, dialyzed again water by using MWCO 100 kDa membran and then freeze-dried to obtain 1.66 g of pure ulvan (named UR). The yield of ulvan was 8.3 % calculated based on algae dried weight.

2.2. Sulfation modification of ulvan

The sulfation of ulvan followed the method of Lihong Fan et al. [9] including two steps: 1) To produce sulfate argent $N(SO_3Na)_3$ by $NaHSO_3$ and $NaNO_2$, 2) To synthesis sulfated ulvan: 0.3 g UR (dissolved in 20 ml distilled water) was added to the sulfate argent solution above under magnetic stirring, the reaction was allowed to proceed for 4 h at 60°C. Then the solution was poured into twice the volume of absolute ethanol by continuous stirring to obtain sulfated ulvan. Obtained ulvan was dissolved in water, dialyzed again water by using MWCO 3500 Da membran and then freeze-dried to obtain pure sulfated ulvan (named URS).

Determination of sulfate content was estimated by the gravimetric method [11]: URS was digested by using 10 ml of HCl 1M for 8 h at 80°C. Then the solution were added excess $BaCl_2$ 1M and barium sulfate precipitate was obtained. The precipitate was washed with warm distilled water to free the Cl^- ion in the precipitate and dried to constant weight (0.187 g). Determination of sulfate content was calculated following the equation:

$$DS(\%) = \frac{\frac{m_{BaSO_4}}{233} x96}{m_{UR}} x100$$

2.3. IR spectra

FT-IR spectra were recorded on an FTIRAffinity-1S Shimadzu spectrometer.

2.4. NMR spectra

NMR spectra were recorded on Bruker AVANCE 500 in D_2O solution using DSS as an internal standard at 70 ºC.

2.5. SEM observation

The SEM images were produced by a Nova NanoSEM 450 EI‑scanning electron microscope at 5 kV.

2.6. Anticoagulant activity

- Chemicals: Dextrose (Sigma) was used as a control sample.
- Animal: BALB/c mice were supplied by Institute of Biological Technology (VAST).
- Method: Put 10 µl of testing samples or dextrose or water into appropriated eppendorfs. The peripheral blood of BALB/c mice was collected from the saphenous vein using a haematocrit tube. Then, immediately add 50 µl of collected blood into sampling prepared eppendorfs. Lightly shake the eppendorf for 2-3 seconds and leave at room temperature. Start to observe the blood clotting at RT condition. Using timer for counting and writing the time-point when blood start to clot.

3. RESULTS AND DISCUSSION

The obtained results on structural determination of ulvan from green seaweed *Ulva reticulata* [7] indicated that the ulvan composed of disaccharide [→ 4)β-D-GlcA(1→ 4)α-L-Rha3S-(1→] (Figure 1). The results of ^{1}H và ^{13}C-NMR analysis were summarized in Table 1.

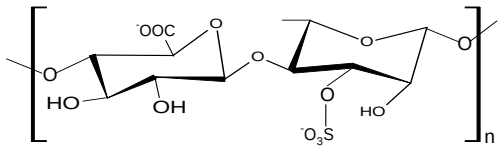


Figure1. Chemical structure of a disaccharide of UR.

Table1. Chemical shifts (ppm) of ^{1}H and ^{13}C-NMR of UR.

	C-1/H-1	C-2/H-2	C-3/H-3	C-4/H-4	C-5/H-5	C-6/H-6
→4)α-L-Rha3S 1→ (A)	100.51/ 4.82	69.71/ 4.20	78.91/ 4.59	78.86/ 3.79	68.89/ 4,13	17.60/ 1.30
→2,4)β-D-GlcA 1→ (B)	103.83/ 4.63	74.58/ 3.35	74.83/ 3.64	79.48/ 3.65	76.70/ 3.82	177.60

The presence of sulfate groups after sulfation process was confirmed by FTIR and NMR spectra.

IR spectra of UR and URS were showed in Figure 2(a, b), respectively. By comparison, the IR spectrum of URS showed absorption peaks at 845 - 880 cm^{-1}, which are characteristics of sulfate groups at axial position. In addition, the intensity of absorption peaks of OH- groups at 3261 - 3370 cm^{-1} were decreased.

From the structure of UR (Figure 1), three positions, which can be sulfated, are C-2 of rhamnose, C-2 and C-3 của glucuronic acid. HSQC spectrum of URS was more complicated than that of UR (Figure 3), the signals were shifted to down field because of the presence of sulfate groups. In HSQC spectrum of URS, the correlation peak C-1/H-1 of rhamnose was splitted and two new correlation peaks of glucuronic acid C-2/H2 at 80.0/3.38 ppm and C-3/H3 at 76.5/3.67 ppm were appeared in the spectrum. In addiction, the intensity of C-2 of rhamnose was decreased. Therefore, we can conclude that three positions C-2 of rhamnose and C-2, C-3 of glucuronic acid were partly sulfated. This result was in agreement with IR spectral analysis above and published work [12].

Under a scanning electron microscope, there were clear differences in surface structure between UR and URS (Figure 4). UR was spongy and cloud-like structure, while, URS was flakey and block-like structure. In URS, the substitution of the hydroxyl groups of the sugar units by sulfate groups may lead to a change in the conformation of the sugar chain, and repulsion between sulfate groups may result in a conformation showing extended or rigid structure.

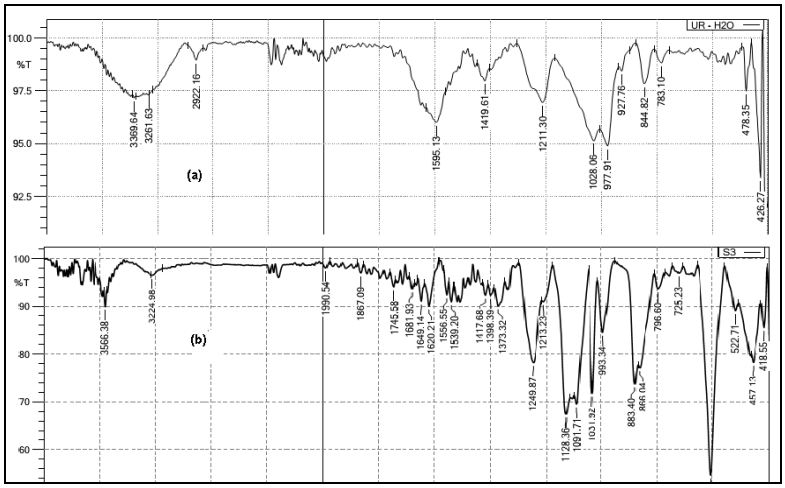


Figure 2. IR spectra of UR (a) and URS (b).

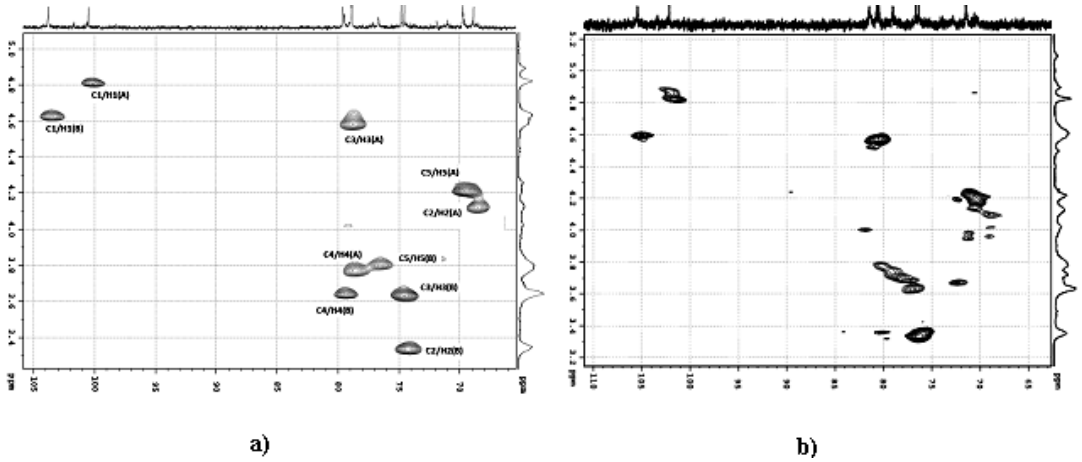
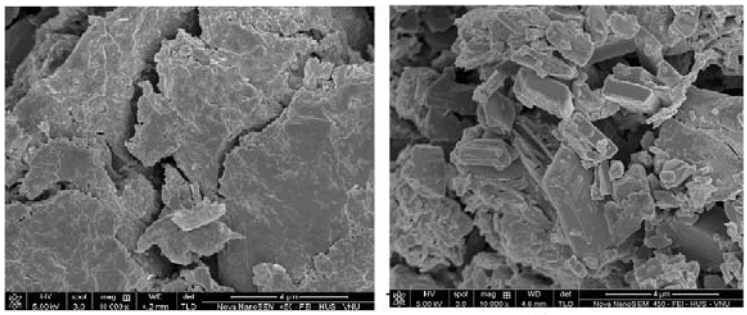


Figure 3. HSQC spectra of UR (a) and URS (b).



a) b)

Figure 4. SEM images of UR (a) and URS (b).

The results of anticoagulant activity of UR, URS and control samples were indicated in Table 2 together with sulfate content.

Table 2. Result of anticoagulant activity.

Sample	Sulfate content (%)	Clotting time (min)
UR	15.04	10
URS	25.68	40
Dextrose		67

The results of anticoagulant activity indicated that the increasing of sulfate content from 15.04 % for UR to 25.68 % for URS resulting in an increasing of bioactivity. Melo et al. [13] reported that the introduction of sulfate to polysaccharides increases the amount of negative charge. Then, the negatively charged sulfate groups can be combined with the positively charged groups from the coagulation protease inhibitor antithrombin, which can activate antithrombin and produce anticoagulant activity.

4. CONCLUSION

Ulvan extracted from green seaweed *Ulva reticulata* was chemically modified to obtain a sulfated derivative. The results indicated that the increasing of sulfate content from 15.04 % for native ulvan to 25.68 % for sulfated derivative was able to change conformational structure and enhance the anticoagulant activity. Our result contributed to confirm the strong relationship between structure and bioactivity.

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TÓM TẮT

NGHIÊN CỨU SỰ THAY ĐỔI CẤU TRÚC VÀ HOẠT TÍNH CHỐNG ĐÔNG TỤ MÁU KHI SULFATE HÓA CỦA ULVAN TÁCH CHIẾT TỪ RONG LỤC *ULVA RETICULATA*

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Ulvan là sulfate polysaccharide có trong thành tế bào của rong lục. Kết quả nghiên cứu cấu trúc của ulvan chiết tách từ rong lục *Ulva reticulata* đã được công bố, trong bài báo này, dẫn xuất sulphate hóa của ulvan được điều chế với mục đích tìm hiểu sự thay đổi cấu trúc và hoạt tính chống đông tụ máu của ulvan. Kết quả chỉ ra rằng khi hàm lượng sulfate tăng thì cấu trúc của ulvan thay đổi và hoạt tính chống đông tụ máu mạnh lên.

Từ khóa: ulvan, rong lục, *Ulva reticulata,* sulfate hóa, cấu trúc, hoạt tính chống đông tụ máu.